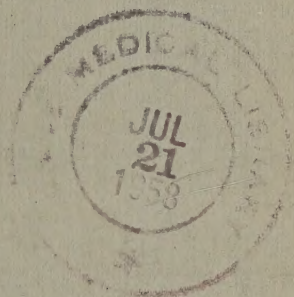


Stockholm
1958

**ON THE PROMOTING EFFECT
OF LYCOPENE AND RELATED SUBSTANCES
ON THE NONSPECIFIC RESISTANCE
OF ANIMALS**

BY
CHRISTIAN LINGEN

WENNER-GREN INSTITUTE
FOR EXPERIMENTAL BIOLOGY,
UNIVERSITY OF STOCKHOLM,
SWEDEN



Diss. med.
Stockholm. Karolinska Institutet
28 MRS 1958

STOCKHOLM 1958

ON THE PROMOTING EFFECT
OF LYCOPENE AND RELATED SUBSTANCES
ON THE NONSPECIFIC RESISTANCE
OF ANIMALS

BY

CHRISTIAN LINGEN

WENNER-GREN INSTITUTE
FOR EXPERIMENTAL BIOLOGY,
UNIVERSITY OF STOCKHOLM,
SWEDEN

STOCKHOLM 1958

MASTERPRINT
FRIDMANS BOKTRYCKERI AB
STOCKHOLM 1958

CONTENTS

	Page
Introduction	5—6
I. Background of the present investigation	7—14
II. Experimental technique	15—19
III. The effects of different lycopene-isomers and related compounds on antibacterial resistance ..	21—25
IV. Influence of various factors on the resistance-enhancing effect of lycopene	27—32
V. Protecting effect of lycopene against repeated infections	33—34
VI. Influence of X-ray irradiation on the effect of lycopene on antibacterial resistance	35—36
VII. Protective effect of lycopene against the development of ascites tumors	37—38
VIII. Lycopene effect and the properdin system	39—41
IX. Effect of lycopene on the level of a resistance-enhancing erythrocyte-stroma factor	43—48
X. Lycopene effect and the Schwartzmann phenomenon	49—50
XI. Some metabolic effects of injected lycopene ...	51
XII. Discussion	53—55
Summary	57—58
Acknowledgements	59
References	61—63



Digitized by the Internet Archive
in 2026

INTRODUCTION

The concept, "nonspecific resistance", or "natural resistance", may be defined as the resistance displayed by an animal against an infection of which it has no earlier experience. This concept has been the subject of intense studies during the last seventy years, the pertinent literature being surveyed in various text-books and reviews (cf. Francis, 1948; Nungester, 1954; Boyd, 1956).

It has been known for a long time that body fluids may possess a bactericidal capacity. Blood serum, for example, has the ability to kill bacteria. This ability disappears when the serum is heated to 56° C for 30 minutes. Phagocytosis plays probably also an important role in natural immunity. The ability of leucocytes to ingest microbes is thought to be due to lytic enzymes present inside these cells.

During the last years these studies have come into a new stage of high activity thanks to the pioneer work of the Pillemer school on the properdin system. An excellent synopsis of the work of this group is found in a recently published *Symposium on Natural Resistance to Infections* (Ann. N. Y. Acad. Sci., Vol. 66, Art. 2, pp. 233—414 (1956)).

The properdin system consists of properdin, all four complements and magnesium. Properdin is a serum protein bound to the third complement. When properdin is removed from serum the latter loses its antibacterial capacity. Yet, properdin itself has no antibacterial effect in itself unless complement and magnesium are present. The Pillemer group has presented evidence that the properdin system is of significance in determining the natural resistance of animals. It has been shown that, upon injection into animals of a yeast autolysate, called zymosan, there ensues a momentaneous decrease of the serum properdin level. When an injection of zymosan is given to rats, followed one hour later by an intraperitoneal injection of *Escherichia coli* (to which rats are naturally resistant) the animals become highly susceptible for these bacteria and die in the infection. If, on the other hand, 24 hours are allowed to elapse after the injection

of the zymosan (under which time there occurs a rise of the properdin level above the initial value) the rats reveal an increased resistance against various, normally pathogenic bacteria.

During a number of decades there has been a search for substances which could stimulate nonspecific resistance. This research has given rise to the so-called nonspecific stimulation therapy; it included various chemicals, X-ray, venesection, etc., all these with more or less success. Attempts have also been made to increase the number of leucocytes, thus enhancing the phagocytic capacity of the organism, or to stimulate the release of natural or acquired antibodies. Petersen (1928) and Perla and Marmorston (1941) have reviewed the earlier literature on clinically tested substances with a resistance-enhancing activity. To be noticed among these substances are animal and plant proteins, protein split products, enzymes, bacterial extracts and vaccines, tissue and leucocytic extracts, and colloidal metals.

In connection with the studies of the Pillemer team on the properdin system some new groups of substances, consisting of bacterial polysaccharides and lipopolysaccharides, have acquired special attention because of their extremely high potency in enhancing nonspecific resistance in animals. Moreover, substances of this type have recently also been found to occur in animal tissues (Landy and Shear, 1957), indicating that they may be involved in the physiological mechanism of nonspecific resistance.

While studying the effects of certain bacterial extracts in enhancing nonspecific resistance in animals the finding was made (Lingen, Ernster and Lindberg, in the press) that the red pigment of tomato, lycopene, could exert a remarkable stimulatory effect on the bacteria to produce the resistance-enhancing principle. It was found, furthermore, that lycopene itself, when injected into animals, is very efficient to enhance nonspecific resistance. In view of the great value the introduction of a chemically well-defined compound into this field of research may be expected to have, it was felt to be of interest to follow up these findings in some detail.

I. Background of the present investigation.

As a background of the present investigation served some clinical observations of cases where injection of broth from cultures of apathogenic bacteria resulted in an improvement of the state of patients suffering of tuberculous meningo-encephalitis. (Lingen, Melin and Enell, 1950). This finding gave rise to the idea that substances produced by apathogenic bacteria might contribute to the nonspecific resistance of a host organism. Some early attempts to test this idea experimentally consisted in injecting rabbits with broths from, or extracts of, cultures of the apathogenic flora of the human mouth cavity and by comparing the antibacterial activities of sera from these rabbits before and after such injections. It was found that after six days of daily repeated injections, the rabbits developed, besides a specific immunity in the classical sense against the bacteria from which the extracts originated, also an increased ability in their blood serum to lyse other bacteria, e. g. *Bacterium tuberculosis* and *Staphylococcus aureus*. This could be shown by following the bacteriolytic effect of rabbit sera, either by means of electron-microscopy, or by using bacteria labelled with P^{32} and measuring the release of P^{32} into the medium (Lingen, 1952).

In order to improve the technique for the preparation of bacterial extracts, one strain of the mouth cavity flora, subsequently identified as *Streptococcus lactis*, was grown in pure cultures and used as starting material. The bacteria were grown in 10-liter bottles. The pH was kept constant at 7.6 by the occasional addition of alkali. Bacteria from 10 bottles were spun down in a Sharpless Ultracentrifuge and suspended in an equal volume of distilled water. Lysis was accomplished by rapid freezing and thawing repeated 10 to 20 times. During this procedure additional amounts of distilled water were

added in order to keep the tonicity low. The extracts were cleared by spinning in the high speed attachment of an International Refrigerated Centrifuge, and reduced to a small volume in the frozen state *in vacuo*. The yellow, somewhat viscous fluid so obtained could evoke, upon injection into rabbits, an enhanced resistance against *S. aureus*. However, these injections had often also toxic effects and therefore a further purification of the extracts was necessary. Among the procedures tested column chromatography on calcium oxide proved to be the relatively best suited method. With the aid of this method it was possible to concentrate the active principle considerably. An interesting feature of the active fraction so obtained was that it showed markedly lipophilic properties although it was soluble in water.

The purified bacterial extracts were tested with respect to their effects on antibacterial resistance, both against *S. aureus* in rabbits, and *Pneumococcus* Type III in mouse. In the case of *S. aureus*, the dose of infection was adjusted so as to cause death within 6—28 hours. In all, 64 rabbits were infected; of these 46 had received, 1—8 days prior to the infection, an intravenous injection of the substance. Of the 46 animals so treated, no effect of the injected substance was obtained in 12 cases; in the remaining 34 cases the rabbits survived the infection, without even showing any signs of sickness. In the case of *Pneumococcus* Type III, two groups of mice, 200 mice in each group, were infected, one of the groups having received an injection of the *S. lactis*-substance 24 hours before infection. 25 hours after the infection there were 80 survivors in the treated group, as compared with 16 survivors in the control group (Lingen, Lindberg and Ernster, 1955).

A great deal of effort has been made to find out means by which the resistance-enhancing effect of the purified bacterial substance could be tested *in vitro*. Since the substance in itself had no antibacterial activity, the next approach consisted in attempting to show an increased antibacterial activity in the blood serum of animals injected with the bacterial substance. When testing the effect of rabbit serum on the growth of *S. aureus* according to the cup method of Fleming, completely negative results were obtained. On the other hand, a rather marked

depression of the respiration of *S. aureus*, as measured with the Warburg technique, could be observed to ensue when serum from rabbits treated 5 days previously with the *S. lactis*-substance was added to the bacterial suspensions. A typical experiment is shown

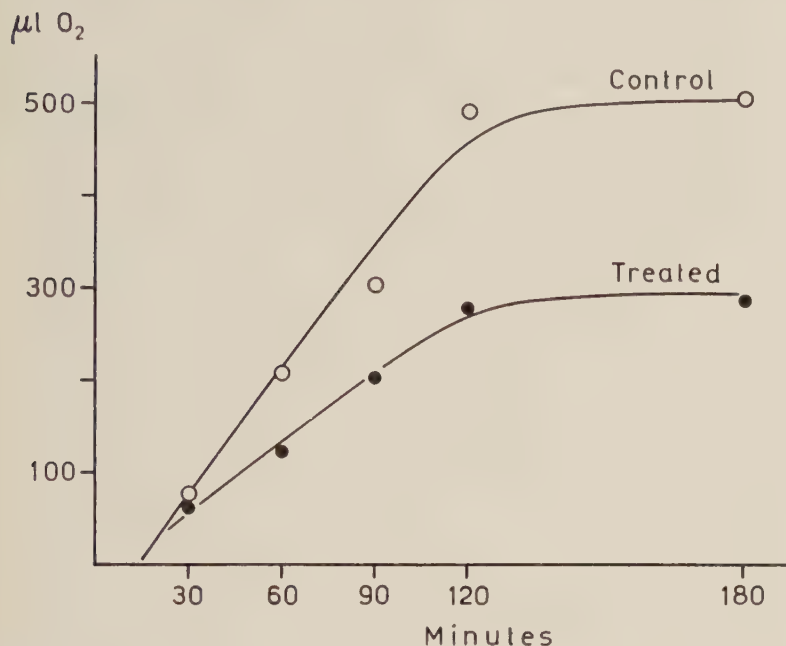


Fig. 1. Effect of different sera on the respiration of *S. aureus*. 10 ml of blood was drawn from a rabbit and the animal was injected with an extract obtained from 1 liter *S. lactis* culture. The serum was kept in CO₂-ice. After 5 days a new blood sample was drawn and the two sera were tested for inhibition of respiration of *S. aureus*. 0.2 ml of serum was added to each Warburg vessel. Each vessel contained bacteria suspended in 2.0 ml physiological saline, 0.2 ml 0.1 M phosphate buffer, pH 6.9, and 0.1 ml 5 % glucose.

in Fig. 1. However, all attempts to obtain a more differentiated picture of this effect, and thus to further elucidate the underlying reaction mechanism, were unsuccessful.

In the course of the Warburg experiments it was observed that the staphylococci had a tendency to agglutinate. This phenomenon was more marked, both stronger and more rapid, the higher was the potency of the tested serum in depressing respiration. There-

fore it appeared possible for a while that the potency of a given serum in agglutinating staphylococci *in vitro* might be used as a measure of the nonspecific antibacterial activity of the serum, and thus, of the resistance-enhancing activity of the injected *S. lactis*-substance tested. Upon closer investigation it turned out, however, that there exists no clear correlation of this sort. One of the difficulties was the repeatedly observed phenomenon that plasma from normal rabbits also normally may agglutinate staphylococci (cf. Birch-Hirschfeld, 1939). Since this phenomenon only occurs with plasma and not with serum, the increased tendency of sera from treated rabbits to agglutinate staphylococci can have been due to a difficulty in making these sera completely free from fibrinogen. Some support for such a possibility was obtained by finding that the injection of the *S. lactis*-substance into rabbits may cause an elevation of the fibrinogen level in plasma. However, not this effect either could be correlated with the state of nonspecific resistance of the animal. All these experiments were done before the first publication of the Pillemer group on the properdin system appeared in 1955. At a later stage of the work, when lycopene had already entered the picture, the effects of the latter on the properdin and complement levels were subjects of a more detailed study. The results of these investigations will be discussed in a later section.

Another initial difficulty was that the yields of active substance from different batches of *S. lactis* varied a great deal. Some batches were highly active, while others showed a relatively low or even no activity. In order to elucidate the reasons for these variations, the conditions used in the cultivation of bacteria were subjected to a detailed study. In the course of these studies the effect of tomato juice was also tested, as this was a constituent of the routinely used culture medium. The surprising finding was then made that the yield of active substance from *S. lactis* could be markedly reduced if the tomato juice was omitted from the culture medium. This is illustrated by the experiment shown in Fig. 2. The Figure compares the respiratory rates of *S. aureus*, in one case incubated in the presence of serum from a rabbit which received substance from streptococci grown without tomato juice, and in the other case with the same

addition but where the substance was obtained from bacteria which had been cultivated in the presence of tomato juice.

As a next step the effect of tomato juice, as injected directly into rabbits, was tested. The tomato juice was filtered free from particulate elements, and 10 ml of the clear yellow solution was

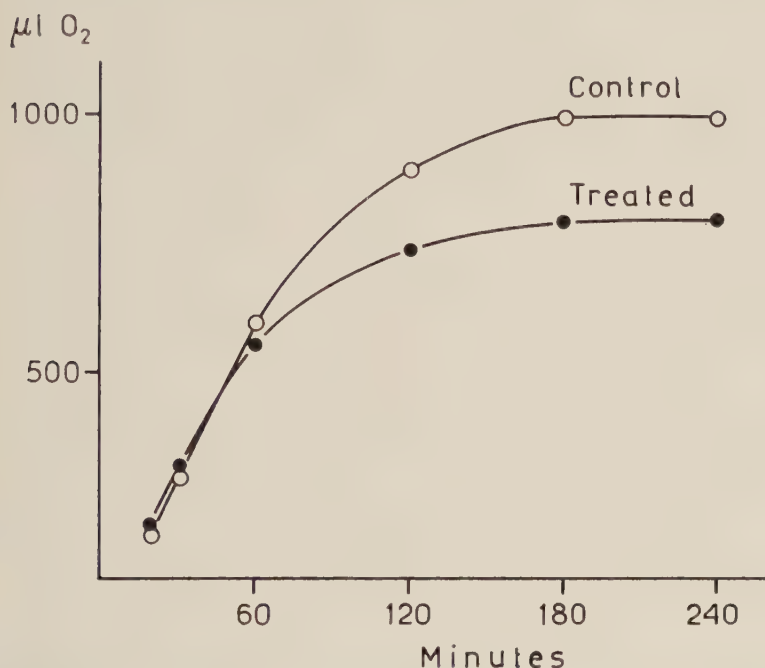


Fig. 2. Effect of different sera on the respiration of *S. aureus*. Two samples of extracts from *S. lactis*. One from bacteria grown on a medium containing tomato juice, and one from bacteria grown on a medium containing no tomato juice. The injected amounts of extracts were equivalent to 1 liter *S. lactis* culture. The samples were injected intravenously into rabbits. After 5 days blood was drawn and serum prepared. Sera tested as in figure 1.

injected intravenously into a rabbit. Fig. 3 compares the effects of rabbit sera, taken before, and 5 days after, the injection of tomato juice, on the respiration of *S. aureus*. Since these findings suggested that the tomato juice itself contains a resistance-promoting factor, the continued work was concentrated on a closer characterization of this factor. The previous observation

that the bacterial substance was markedly lipophilic directed the attention to the lipophilic constituents of the tomato fruit. It was soon found that the activity could easily be removed from the

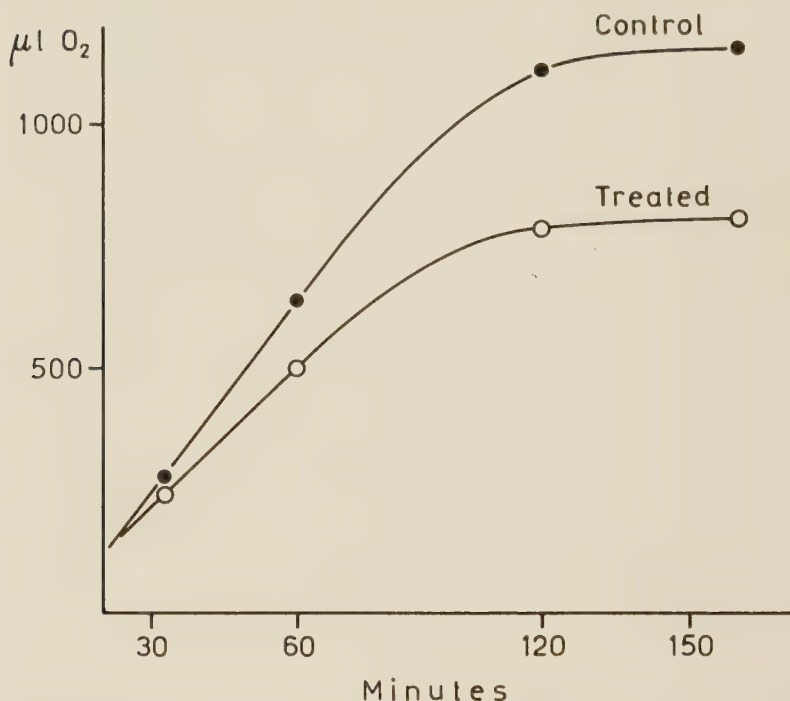


Fig. 3. Effect of different sera on the respiration of *S. aureus*. Procedure as in figure 1 but bacterial extract was replaced by tomato juice. 10 ml of filtered tomato juice was injected intravenously into the rabbit.

tomato juice by extraction with petroleum ether, and that the petroleum ether extract so obtained could account for the resistance-promoting effect. These extracts were bright yellow and gave a spectrum characteristic of lycopene. Therefore, lycopene was isolated from tomato juice (cf. Chapter II), suspended in Tween-80, and injected into rabbits. Five days after the injection of lycopene the rabbits were infected with a massive dose *S. aureus*. Some typical results are shown in Table I. The lycopene-treated rabbits revealed a markedly increased resistance

against the infection as compared with the controls. Furthermore, the sera of these rabbits were able to inhibit respiration of *S. aureus in vitro*, although, again, there was no clear correlation between this effect and antibacterial resistance.

At this stage it became desirable to work with a larger number of animals, in order to obtain a more quantitative picture of the

Dose of lycopene μ g	Number of rabbits	Survivors after		
		24 hours	48 hours	96 hours
0	6	0	0	0
800	3	3	2	2
1600	3	3	2	2

Table I. Effect of lycopene on resistance against *S. aureus* in rabbits. Lycopene was injected 5 days before challenge.

lycopene effect. For this purpose experiments were started with mice, and using, to begin with, *Pneumococcus* Type III as test bacteria. White heterozygote mice weighing 15–25 g. were divided into groups of 10. Eleven groups were injected intraperitoneally with 1 mg lycopene per mouse, representing eleven different batches of lycopene, and four groups served as controls. 24 hours after the injection of the test groups each mouse in all groups received a large dose of *Pneumococcus* Type III. As seen in Table II, all the lycopene-treated groups showed a prolonged survival. However, the survival times between the individual groups were greatly varying, indicating that the various lycopene batches were not exactly identical with respect to their resistance-promoting potency. To investigate this problem more closely, first of all a better test system was needed, since in the pneumococcus-test the difference in survival time between lycopene-treated mice and controls was too small to allow a more quantitative evaluation.

In the meantime a number of reports had appeared in the literature on studies of the effects of various polysaccharides on the nonspecific resistance of mice. In most of these studies *Klebsiella pneumoniae* was used as test bacterium. Since it was to

presume that the effects of these polysaccharides and that of lycopene might be ultimately due to a common mechanism (see

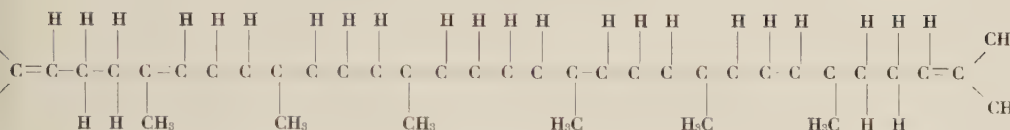
Group number	Number of mice	Survivors after					
		24	25	26	28	30	36
hours following infection							
1	10	9	8	6	4	4	0
2	10	2	2	2	1	1	0
3	10	8	8	5	3	3	0
4	10	7	7	6	4	3	0
5	10	6	5	4	2	2	0
6	10	9	8	8	6	5	0
7	10	9	8	8	7	6	0
8	10	3	2	1	1	1	0
9	10	4	4	4	2	2	0
10	10	3	2	2	2	2	0
11	10	6	5	4	4	3	0
Control	40	4	4	0	0	0	0

Table II. The resistance promoting effect of lycopene against pneumococcus Type III in mice. 1 mg of natural lycopene per mouse was given 24 hours before challenge. 11 groups of mice, each group injected with a different batch of lycopene. 40 mice in the control group.

Discussion) it appeared reasonable to introduce *Klebsiella p.* as test bacterium also in the present work. In the subsequent chapters a more detailed account is given of the results hitherto obtained concerning the effects of lycopene on the antibacterial resistance of mice as studied with *Klebsiella p.* In addition, some data are included on the effects of lycopene in promoting resistance against certain types of ascites tumors in mice.

II. Experimental technique.

Preparation of lycopene. Lycopene ($C_{40}H_{56}$),



was prepared by following the procedure described by Sandeval and Zechmeister (1949), with minor modifications. The routine procedure was as follows: To the contents of 20 tins of tomato juice Del Monte (1 lb per tin) 10 liters of 96 % ethanol was added. The mixture was filtered through gauze and the residue was suspended in 2 liters of absolute ethanol and centrifuged. After decanting the ethanol, the sediment was spread out on sheets of filter paper and dried in a thermostate at $+40^{\circ}\text{C}$ for 24 hours. The dry product was ground to a fine powder and extracted with several portions of carbon disulfide until the remaining powder was nearly colorless. Usually about 500 ml of carbon disulfide was needed in total. The dark-red carbon disulfide solution was then put to evaporation at $+40^{\circ}\text{C}$ in a carbon dioxide atmosphere. When only about 40 ml of solution was left a thick crystal crust of lycopene started to precipitate. At this stage 1 liter of absolute ethanol was added and the mixture was shaken vigorously. It was then let to stand at -20°C for 6 hours, after which the crystals could be separated in a sintered glass filter. The crystal mass was washed twice with ice-cold absolute ethanol. The product so obtained, usually about 500 mg, could be used, either directly, or after recrystallization from hot petroleum ether.

The preparation was checked in the Beckmann spectrophotometer and found to give a spectrum identical with that described by Karrer and Jucker (1948) for *all-trans*-lycopene. Possible traces of *cis*-forms of lycopene could be successfully eliminated by recrystallization from petroleum ether. It was also found advisable to perform the preparation under protection from light.

Isomers of lycopene were prepared by treating the *all-trans*-form with iodine in the presence of light according to Trombly and Porter (1953). Single isomers could be separated by means of chromatography on calcium oxide. Recently synthetic lycopene has become commercially available. The synthesis is made

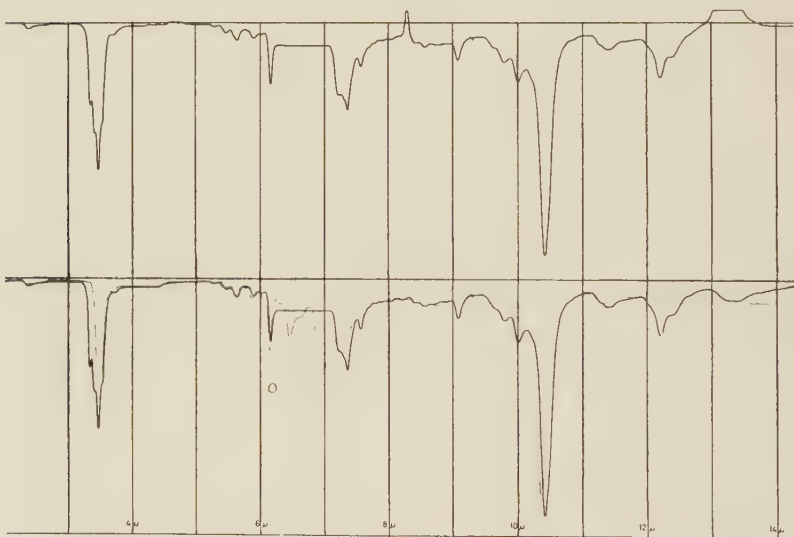


Fig. 4. Comparison of infrared spectra of natural and synthetic lycopene. Solvent CS_2 .

according to the method of von Isler, Gutmann, Lindlar, Montavon, Rüegg, Ryser and Zeller (1956). A comparison between the infrared spectra of the synthetic compound and of that obtained from tomato by the above procedure is made in Fig. 4. It shows that the two compounds are identical.

Preparation of suspensions of lycopene for injection. Stock suspensions of lycopene were prepared as follows: 100 mg lycopene was triturated in 2 ml Tween-80 at room temperature until a homogenous paste was obtained. Physiological saline at room temperature was added dropwise under vigorous stirring to a final concentration of 10 mg lycopene per ml suspension. 0.1 ml suspension was injected per mouse, either directly, or after dilution with a 4:1 mixture of saline and Tween-80.

Bacterial cultures. Two strains of *Klebsiella p.* were used, the highly virulent AD strain¹ with an LD₅₀ of the order of magnitude of 10 cells or less, and a less virulent strain with an LD₅₀ ranging between 10⁴ and 10⁶ cells. The bacteria were grown on blood agar plates and transferred to ordinary broth at 37° C 24 hours before use. Each mouse received an intraperitoneal injection of 0.2 ml broth containing the desired dose of bacteria.

The amount of bacterial cells was determined with the aid of Opacity tubes from Burroughs Wellcome & Co. Ltd., London. This determination gave 5×10^8 cells per ml culture broth with both strains after cultivation for 24 hours at + 37° C.

Test animals. The experiments were performed with mice and rabbits. White, heterozygote mice weighing 18—25 g. were used throughout the experiments. The weight of the rabbits was 1.5—2 kg. The animals were kept on a standard laboratory diet.

Ascites tumor cells. Ascites tumor cell material was obtained by puncture of tumor-bearing mice from the stocks of Prof. G. Klein, Dept. of Tumor Biology, Karolinska Institutet, Stockholm. Cell counting was made according to Schrek (1936). Inoculation was made intraperitoneally, after dilution of the cell suspensions to desired concentration with physiological saline.

¹I am indebted to Dr. J.S. Kiser, American Cyanamid Company, Pearl River, N. Y., for kindly sending me a sample of *Klebsiella pneumoniae* AD.

Expt no	Number of mice per group	Time after infection, hours	Number of survivors		
			Lycopene treated groups		Control group
			Natural lycopene	Synthetic lycopene	
93	10	17	10		9
		18	10		8
		21	10		7
		22	10		6
		24	10		5
		48	10		1
		96	10		1
		120	10		1
		144	10		1
94 a	10	17	10		10*)
		19	10		8
		21	10		6
		23	10		3
		24	10		1
		48	10		0
94 b	10	17	10		10*)
		19	10		10
		21	10		10
		23	10		8
		24	10		4
		48	10		3
94 c	10	17	10		9*)
		19	10		9
		21	10		8
		23	10		4
		24	9		0
		48	9		0
94 d	10	17	9		9*)
		19	9		6
		21	9		4
		23	9		1

*) Treated with 0.02 ml Tween-80 24 hours before infection.

Expt no	Number of mice per group	Time after infection, hours	Number of survivors		
			Lycopene treated groups		Control group
			Natural lycopene	Synthetic lycopene	
94 e	10	24	9		1
		48	9		1
		17	10		7*)
		19	10		7
		21	10		7
		23	10		4
		24	9		1
		48	9		1
153	6	19	6	6	5
		23	6	6	4
		48	6	6	0
179	6	16	6		0
		96	5		0
		120	5		0
220	6	23		6	0
		28		6	0
		48		4	0
		23		6	0
		28		6	0
		48		6	0
		23		6	0
		28		6	0
223	6	48		6	0
		23		4	0
		48		4	0
227	6	72		4	0
		19	6	6	2

*) Treated with 0.02 ml Tween-80 24 hours before infection.

Table III. Effect of natural and synthetic lycopene on resistance of mice against *Klebsiella p.* of low virulence. 10 or 6 mice in each group. 1 mg lycopene was given intraperitoneally 24 hours before challenge.

III. The effects of different lycopene-isomers and related compounds on antibacterial resistance.

In Table III a number of typical experiments are summarized, illustrating the effect of lycopene on the resistance of mice against *Klebsiella p.* In all these experiments each mouse had received an intraperitoneal injection of 1 mg lycopene 24 hours before infection. The Table includes experiments done with both natural and synthetic lycopene.

It has been observed at an early stage of the present study that the increased resistance in animals, ensuing upon the injection of lycopene could vary depending on the way in which the preparation of the lycopene from tomato was made. As mentioned previously, it seemed to be of special importance to protect the substance against light. This pointed to the possibility that the *all-trans*-form of lycopene might be more potent in enhancing nonspecific resistance than are the *cis*-forms, which are known to be formed from the *all-trans*-form upon the action of light. By preparing *cis*-isomers of lycopene according to the method of Trombly and Porter (1953), it was possible to obtain support for this view. As seen in Fig. 5 the isomerised preparation gave markedly lowered survival as compared with the starting *all-trans* compound. The difference could be further accentuated by removing the small amount of *all-trans*-lycopene from the reaction mixture by chromatography on calcium oxide. It can thus be concluded that *all-trans*-lycopene possesses a higher resistance-enhancing potency than its *cis*-isomers.

A problem of interest was to investigate to what extent compounds chemically related to lycopene are able to induce a similar effect. In Tables IV—VI some such comparative experiments are shown. As emerges from these Tables, lycopene was superior in promoting resistance against *Klebsiella p.* in mice

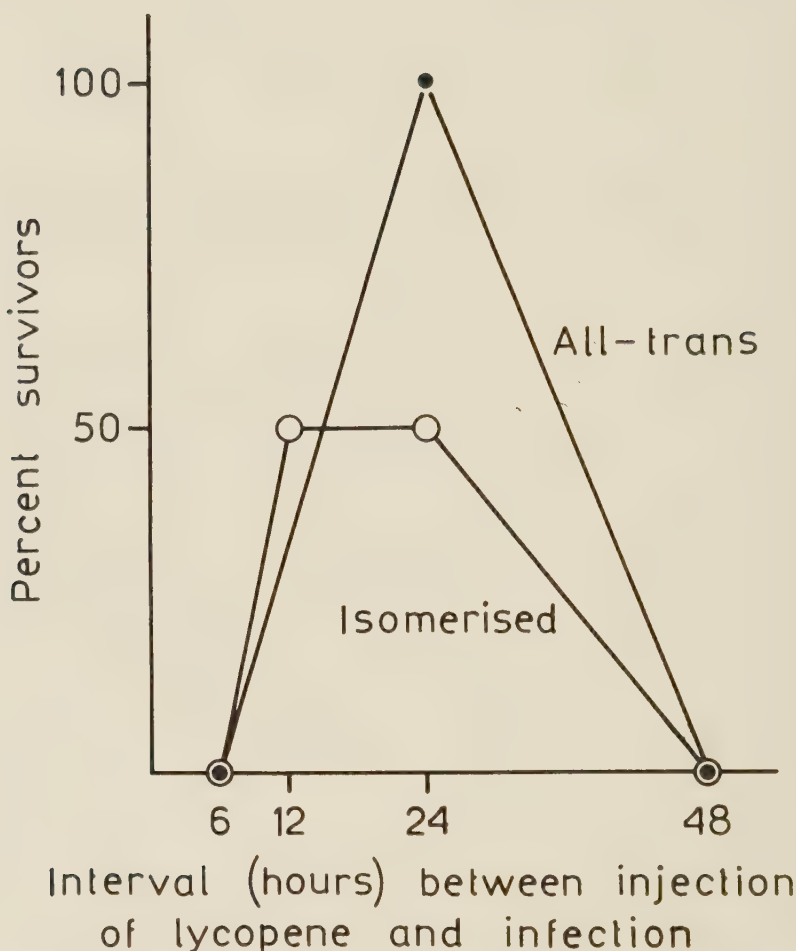


Fig. 5. Resistance-promoting effect of *all-trans* and *cis-isomers* of lycopene. 6 mice in each group. Challenge with *Klebsiella p.* of low virulence 6, 15, 24 and 48 hours after injection of 1 mg of the *cis-isomer* and 6, 24 and 48 hours after injection of the *all-trans-isomer*. Dose of infection 10^9 cells.

when compared with the following compounds²: crocetin,

² I am greatly indebted to the following persons for generous gifts of various compounds: Dr. P. Karrer, Chemisches Inst. d. Universität, Zürich, Switzerland (crocetin); Dr. H. J. Bielig, Inst. f. Chemie, Heidelberg, Germany (crocetin); Dr. F. W. Quackenbusch, Purdue University, Lafayette, Indiana (phytoene); and Dr. N. A. Sørensen, Norges Tekniska Høgskole, Trondheim, Norway (squalene). Bixin and synthetic and natural β -carotene were purchased from Hoffman-La Roche & Co., Bale, Switzerland.

Dose in micrograms		Survivors after 24 48 hours following infection	
Crocetin	100	0	0
Crocetin	1000	6	6
Crocin	1000	0	0
Lycopene	100	6	6
Lycopene	1000	6	6
Control		0	0

Table IV. Effect of natural lycopene, crocetin and crocin on resistance of mice against *Klebsiella p.* of low virulence. 6 female mice in each group. The substances were given i.p. 24 hours before challenge.

Substance tested	Dose in microgram	Survivors after 24 48 hours following infection	
Lycopene	500	6	6
Natural carotene			
25 % α 50 % β 5 % γ	1000	2	2
Natural carotene +	1000		
lycopene	100	6	6
Zymosane	3000	6	6
Vitamin A	300	0	0
Squalene	1000	0	0
Phytoene	1000	0	0
Control		1	0

Table V. Effect of lycopene and various other substances on resistance of mice against *Klebsiella p.* of low virulence. 6 female mice in each group. 18 female mice in the control group. The substances were given intraperitoneally 24 hours before challenge.

carotene, bixin, phytoene, squalene, Vitamin A. Noteworthy is that all of these compounds, except those which are oils (phytoene, squalene, Vitamin A), had a definite activity, even

Compound and dose in micrograms		Number of mice	Survivors after 17 21 49 91 139 169 hours following infection					
None			12	12	11	3	3	3
Lycopene (synthetic)	20	6	6	3	0	0	0	0
	100	6	5	5	3	3	3	3
	500	6	6	6	5	5	5	4
	1000	6	6	6	6	5	5	5
Bixin	20	6	6	5	1	0	0	0
	100	6	6	4	0	0	0	0
	500	6	6	4	1	0	0	0
	1000	6	5	5	4	4	4	4
β -Caroten (synthetic)	20	6	5	4	0	0	0	0
	100	6	6	4	0	0	0	0
	500	6	6	6	2	2	2	2
	1000	6	6	6	4	4	4	4

Table VI. Comparison between the resistance-enhancing effects of different carotenoids.

Challenge with *Klebsiella* p. of low virulence 24 hours after i. p. injection of the test substance.

if this was slight compared with that of lycopene. The problem as to whether this depends on the fact that the crystalline state is of significance for the resistance-promoting activity will be discussed in a later section.

It should be pointed out also that a comparison of the various substances with respect to their biological activity in relation to lycopene and to each other may be valid only under the experimental conditions used in the present experiments. Variations in dose, especially in relation to the time interval applied between injection of the substance and the infection, may be expected to influence the patterns of action of the individual

substances in the same way as it does in the case of lycopene (cf. Chapter IV, Table VII). Whether these patterns are identical for different compounds or vary from one compound to another, thus changing their relative activities from one set of conditions to another, is not yet investigated.

IV. Influence of various factors on the resistance-enhancing effect of lycopene.

Time of injection. The time of injection of lycopene in relation to the time of challenge proved to be of great significance for the attained effect. Fig. 6 illustrates this relationship. In the experiment shown in this Figure maximum effect was obtained when lycopene was given 24 hours before infection. Although this time could vary slightly from one experiment to another, no exception was found from the rule that lycopene only had an effect if it was given before the infection, and also, that at least six hours have to elapse between the injection of lycopene and the bacterial infection in order to obtain a significant effect.

Dose of lycopene. The influence of the dose of lycopene on the obtained effect is shown in Table VII. In this experiment two different batches of synthetic lycopene were given to two parallel groups of mice. As emerges from the Table, the dose relationships may vary from one batch of lycopene to another. The batches differed in the present case in that one of them was easy to disperse in Tween-80 to a homogenous microcrystalline suspension, while the other consisted of relatively coarse crystals difficult to suspend. In the first case optimal effects were obtained with doses between 0.03 and 0.5 mg with a clear decline of the effect at a dose of 1 mg. In the second case, no optimum was attained until at a dose of 0.125 mg. but, on the other hand, there was no decline at a dose of 1 mg. In both cases, a dose as low as 0.002 mg gave a slight but significant effect. These findings indicate that the rate of resorption of lycopene may have an inverse relation to its resistance-enhancing effect. A similar conclusion has recently been arrived at by Forssberg (personal communication) in studies of the protective effect of lycopene

against X-ray irradiation. It appears, furthermore, that batches of lycopene of easily dispersible crystalline structure may give toxic effects more readily than those which are difficult to

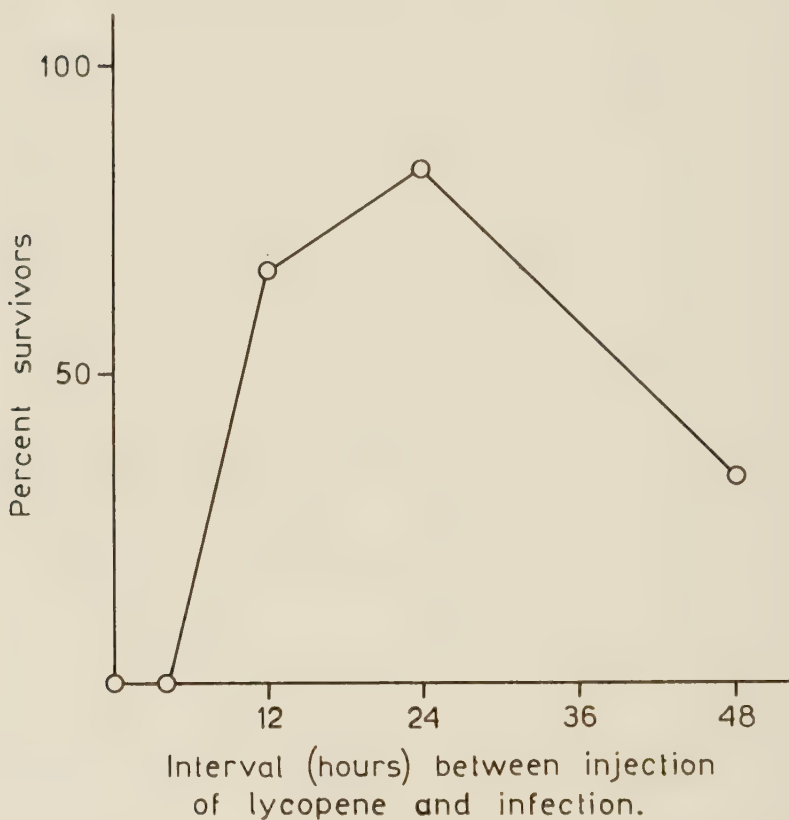


Fig. 6. Influence of time for lycopene injection in relation to time of challenge on the development of resistance against the lowly virulent *Klebsiella p.* strain. Dose of infection 10^9 cells.

disperse. Concerning toxicity it may also be mentioned that while mice reveal a relatively high tolerance against lycopene, withstanding doses up to 5 mg per mouse without showing any external signs of intoxication, rabbits are extremely sensitive to this compound. It has repeatedly been found that an intravenous injection of 15 mg of lycopene into a rabbit weighing

Hours after infection	Bacterial dilutions										
	10^{-0}	10^{-2}	10^{-4}	10^{-5}	10^{-7}	10^{-8}	10^{-10}	10^{-12}	10^{-4}	10^{-5}	10^{-8}
0	6	6	6	6	6	6	6	6	6	6	6
2	6	6	6	6	6	6	6	6	6	6	6
4	3	6	6	6	6	6	6	6	6	6	6
6	0	6	6	6	6	6	6	6	6	6	6
8		6	6	6	6	6	6	6	6	6	6
10		5	6	6	6	6	6	6	6	6	6
12		5	6	6	6	6	6	6	6	6	6
14		4	6	6	6	6	6	6	6	6	6
16		3	6	6	6	6	6	6	6	6	6
18		3	6	6	6	6	6	6	6	6	6
20		3	5	6	6	6	6	6	6	6	6
22		2	5	6	6	6	6	6	6	6	6
24		2	5	6	6	6	6	6	6	6	6
26		2	5	6	6	6	6	6	6	6	6
28		2	5	6	6	6	6	6	6	6	6
30		2	5	6	6	6	6	6	6	6	6
32		2	5	6	6	6	6	6	6	6	6
34		2	5	6	6	6	6	6	6	6	6
36		2	5	6	6	6	6	6	6	6	6
38		2	5	6	6	6	6	6	6	6	6
40		2	5	6	6	6	6	6	6	6	6
42		2	5	6	6	6	6	6	6	6	6
44		2	5	6	6	6	6	6	6	6	6
46		2	5	6	6	6	6	6	6	6	6
48		2	5	6	6	6	6	6	6	6	6
50		2	5	6	6	6	6	6	6	6	6
52		2	5	6	6	6	6	6	6	6	6
54		2	5	6	6	6	6	6	6	6	6
56		2	5	6	6	6	6	6	6	6	6
58		2	5	6	6	6	6	6	6	6	6
60		2	5	6	6	6	6	6	6	6	6
62		2	5	6	6	6	6	6	6	6	6
64		2	5	6	6	6	6	6	6	6	6
66		2	5	6	6	6	6	6	6	6	6
68		2	5	6	6	6	6	6	6	6	6
70		2	5	6	6	6	6	6	6	6	6
72		2	5	6	6	6	6	6	6	6	6
74		2	5	6	6	6	6	6	6	6	6
76		2	5	6	6	6	6	6	6	6	6

Table VIII. Effect of lycopene on resistance of mice against varying doses of *Klebsiella* p.AD (dilutions 10^{-0} — 10^{-8}). Infections made 24 hours after intraperitoneal injection of 500 micrograms of lycopene.

1.5 kg causes a grave shock leading to death within a few minutes. This effect, like the enhancement of resistance, seems to be greatest with the *all-trans*-isomer of lycopene.

Sample	Dose of lycopene μg	Survivors after hours							
		6	14	19	24	29	35	48	96
—	—	10	7	0					
A	2	10	9	5	5	3	2	2	2
B	2	10	9	7	5	4	3	1	1
A	4	10	9	5	5	3	2	2	2
B	4	10	9	8	5	5	5	5	5
A	8	10	9	9	3	2	2	2	2
B	8	10	10	8	2	1	1	1	1
A	16	10	10	8	3	1	1	1	1
B	16	10	9	6	3	3	3	3	3
A	32	10	10	10	6	5	5	4	4
B	32	10	10	10	8	8	8	8	7
A	64	10	10	10	6	5	5	5	5
B	64	10	10	10	9	9	9	9	7
A	128	10	10	10	9	9	9	9	9
B	128	10	10	10	9	9	9	9	9
A	256	10	10	10	10	10	10	10	10
B	256	10	10	9	8	8	7	7	7
A	512	10	10	10	10	10	10	10	10
B	512	10	10	10	9	9	9	9	9
A	1024	10	10	10	10	10	10	10	10
B	1024	8	7	6	3	3	3	3	3

Table VII. Effect of varying doses of synthetic lycopene from two different batches, A (lot No. B 508306) and B (lot No. B 510126) on resistance of mice against *Klebsiella p.* of low virulence. 10 female mice in each group. Lycopene was given intraperitoneally 24 hours before challenge.

Dose of bacteria. Table VIII shows an experiment where the dose of bacteria was varied. In this experiment the highly virulent AD strain of *Klebsiella p.* was used. The dose of lycopene

was constant throughout all groups, each mouse receiving a single intraperitoneal injection of 0.5 mg lycopene 24 hours before challenge. Although the data do not allow an exact quantitative evaluation, e. g. in terms of LD₅₀, of the obtained stimulation of the antibacterial resistance, it is obvious from the Table that the lycopene-treated mice revealed a survival curve with a bacterial dilution of 10⁻⁴ which was similar to, or even better than, the curve obtained with the control group receiving a bacterial dilution of 10⁻⁷. This would mean, roughly, an improvement of the resistance by a factor of 1000.

Route of administration. Table IX gives a comparison of the effects of lycopene administered by different routes. As it is seen, intraperitoneal injection has given the best results, followed

Route of injection and dose in microgram		Survivors after	
		24 hours following infection	48
Intraperitoneal	100	6	6
Intravenous	100	5	3
Intramuscular	100	5	0
Control		0	0

Table IX. Effect of the route of administration on the resistance-promoting effect of lycopene. 6 mice in each group. Challenge with an intraperitoneal injection of *Klebsiella p.* of low virulence 24 hours after lycopene injection.

by the intravenous and intramuscular routes of administration. In all three cases, and independently of the dose, optimal effects occurred when the lycopene was given 24 hours before the infection. No resistance-promoting effect of lycopene was obtained when the substance was given *per os*. This could be expected on the basis of the known fact that lycopene (in contrast to carotene) is not resorbed by way of the gastro-intestinal system (Gillam and Kon, 1940).

Sex and age. In the course of the above studies on dose relationships, it was observed that a difference may prevail between

males and females with respect to their responsiveness to lycopene. This is illustrated by the experiment in Table X. It emerges from this Table that lycopene is more effective in promoting resistance in adult females than in old females or in adult males.

Description of mice	Number of mice	Lycopene treatment	Survivors after	
			24 hours of infection	48
Young females, 12—15 g	6	—	0	0
	6	+	3	3
Old females, 20—25 g	6	—	0	0
	6	+	6	6
Young males, 12—15 g	6	—	0	0
	6	+	6	6
Old males, 20—25 g	6	—	0	0
	6	+	3	3

Table X. Dependence of the lycopene-induced resistance against *Klebsiella p.* on the sex and age of mice.

A sex dependence similar to that reported here has also been observed in studies concerning the protective effect of lycopene against X-ray challenge (Forssberg and Lingen, to be published). It was found that 1.5—2 mg of lycopene given to female mice 24 hours before irradiation protects against the radiation damage while the same dose of lycopene given under the same conditions to males is an overdosage, even accelerating death.

V. Protecting effect of lycopene against repeated infections.

As was pointed out previously, the effect of lycopene in elevating the antibacterial resistance of mice reaches its maximum

Time in hours of FIRST INFECTION REINFECTION in relation to time of lycopene treatment		Number of survivors after 24 96 hours following last infection	
24	48	6	6
48		1	1
24	72	6	6
72		0	0
24	96	6	6
96		0	0
24	120	6	6
120		0	0
24	144	4	4
144		0	0
24	192	2	2
192		0	0
24	216	0	0
216		0	0

Table XI. Effect of infection on the duration of resistance against *Klebsiella p.* after lycopene injection. 90 mice were injected with 1 mg lycopene per mouse. 24 hours later 45 mice were infected with *Klebsiella p.* of low virulence. Surviving animals (42) were divided into 7 groups with 6 mice in each group. One group of 6 of those lycopene-treated mice which had not been challenged 24 hours after the lycopene-treatment served as controls to each surviving group. The 7 groups with controls were then infected after different lengths of time.

after about 24 hours. After this time the effect decreases and it is exhausted usually after 72 hours. It has been observed, however, that mice which once survived an infection given 24 hours after the injection of lycopene retain their elevated resistance against repeated infections for 7 days, or even longer. Table XI shows an experiment illustrating this phenomenon. The role of repeated challenge in prolonging the effect of lycopene is not clear. A similar observation has been made in experiments with Ehrlich ascites tumors, where it was found that lycopene-treated mice once surviving the consequences of the inoculation of 100 cells can be re- and re-inoculated for up to six months, and with doses amounting to millions of cells, without developing any tumors.

VI. Influence of X-ray irradiation on the effect of lycopene on antibacterial resistance.

The inhibitory effect of X-rays on the formation of antibodies has been described by several authors (cf. Dixon, Talmage and Maurer, 1952; Marcus, Donaldson and Esplin, 1955). In spite of this, relatively little is known about the mechanism of this inhibition. An X-ray dose of 400—600 r, given 48 hours before the injection of antigens, is known to completely suppress the

X-ray dose r	Lycopene- treatment	Percent survivors after 48 hours following infection
0	—	0
0	+	100
100	+	80
300	+	100
900	+	90
1000	+	80
2000	+	60
3000	+	0

Table XII. Effect of different doses of X-ray on the enhanced resistance of mice as induced by 1 mg of lycopene given 24 hours before irradiation and infection with *Klebsiella p.* of low virulence.

formation of antibodies. However, if the irradiation is made simultaneously with, or 6 hours after, the administration of antigen it has no major effect on the formation of antibodies.

On the basis of these facts, and of the observations that lycopene exhibits a protective effect against total body irradiation

tion (Forssberg and Lingen, to be published), it was of interest to investigate the influence of X-ray irradiation on the effect of lycopene on antibacterial resistance. Table XII shows an experiment in which the influence of varying doses of X-ray was investigated. Irradiation was made 24 hours after the injection of lycopene. Immediately after irradiation, the animals were infected with *Klebsiella p.* As emerges from the Table, doses of X-ray ranging between 100 and 1000 r had no effect on the lycopene-induced enhancement of the antibacterial resistance of the animals. Beyond 1000 r there was a clear decrease, and at 3000 r the lycopene effect was completely absent.

Time of lycopene injection in relation to time of irradiation	Survivors after 48 hours, in percent
24 hours before irradiation	80
3 " " " 	80
24 " after " 	80
48 " " " 	20
72 " " " 	0

Table XIII. Sensitivity of the lycopene-induced resistance to X-ray irradiation. 10 female mice in each group.

Challenge with *Klebsiella p.*, low virulence, 24 hours after intraperitoneal injection of 1 mg lycopene.

In the experiment shown in Table XIII, lycopene was injected at various times in relation to the time of irradiation, the X-ray dose being fixed at 400 r. Lycopene was given 24 hours or 3 hours before, or 24, 48 or 72 hours after, irradiation. In all cases, the bacteria were injected 24 hours after the injection of lycopene. The results show that X-ray irradiation can eliminate the effect of lycopene on antibacterial resistance also at low doses, provided that the lycopene is given at least 48 hours after irradiation.

VII. Protective effect of lycopene against the development of ascites tumors.

Fig. 7 illustrates the protective effect of lycopene against the development of Ehrlich ascites tumors in mice. As seen in the Fig., positive results could be obtained also when the lycopene

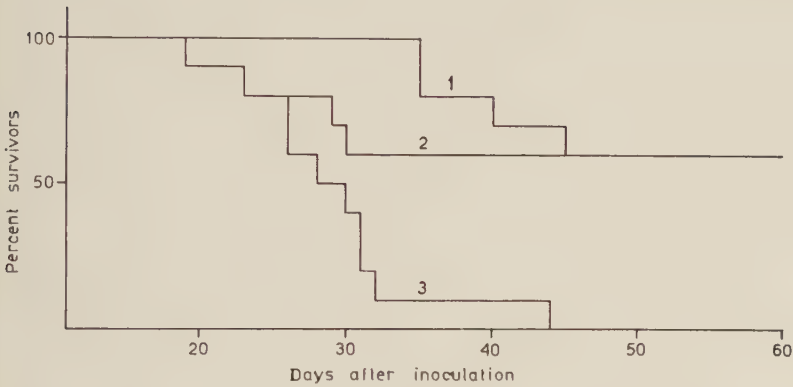


Fig. 7. Effect of lycopene on the development of resistance against Ehrlich ascites tumor. 10 mice in each group. 1 mg lycopene given intraperitoneally to each mouse.
 1. Inoculum dose 10000 cells i. p. Lycopene injected 24 hours later.
 2. " " 1000 " " " " 3 days "
 3. " " 10000 " " " " "

was injected after, rather than before, the inoculation of the tumors, provided that the inoculum dose was not higher than 1000 cells per mouse, and the tumor had not yet reached a size exceeding 10,000 cells at the time of the lycopene injection. When, on the other hand, lycopene was injected into mice with already advanced ascites tumors, it had no effect on the further growth of the tumors.

Results similar to those shown above were obtained with certain other types of ascites tumors while still others failed to respond. A number of solid transplantable mammary carcinomas, growing in their own inbred strain of origin, remained also uninfluenced by the treatment. The experiments gave the impression that only certain tumors growing in genetically foreign hosts can be inhibited.

VIII. Lycopene effect and the properdin system.

In view of the suggested involvement of properdin in the mechanism of nonspecific resistance (Pillemer, Blum, Lepow, Ross, Todd and Wardlaw, 1954), it was of interest to investigate how lycopene influences the serum properdin level of animals. After injection of lycopene into rabbits or mice an almost momentaneous drop of the properdine level could be observed,

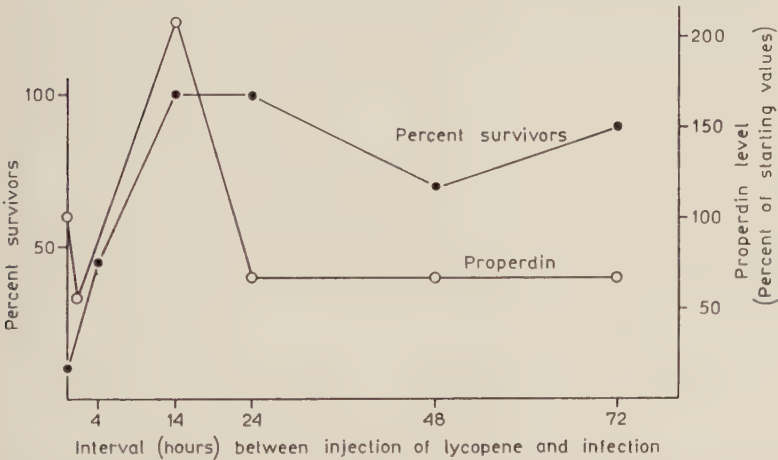


Fig. 8. Relation between properdin level and nonspecific resistance. 10 mice in each group. Challenge with *Klebsiella* p. of low virulence. Properdin values in relative units. Dose of infection 10^9 cells.

followed by a marked rise above the level prevailing in the animal before the lycopene injection. In rabbits injected intra-venously with 10 mg of lycopene, maximum properdin levels were obtained about 5 days after injection. In mice the corresponding time was about 20 hours. Fig. 8 shows an experiment

where the properdin level was followed in mice after the intra-peritoneal injection of 1 mg lycopene. The development of the increased resistance of the mice is also shown. As seen in the Fig., the maximum properdin level occurs almost simultaneously with the maximum resistance. Yet, the properdin level falls relatively abruptly to values which lie under the normal value and still there is at least in this particular experiment, a clearly increased resistance even after 72 hours. The above findings are similar to those described by Landy and Pillemer (1956 a, b) for zymosan and bacterial lipopolysaccharides.

Lycopene					
Rabbit number	C'1	C'2	C'3	C'4	C'
122	1.8	1.2	1.5	—	1.5
101	2.8	1.3	—	—	2.8
118	2.0	1.4	1.7	—	1.4
119	6	—	1.6	—	1.5
117	1.7	1.1	1.4	1.3	—
Zymosan					
120	1.6	1.2	1.2	—	1.8
122	2.1	1.4	1.7	—	2.2

Table XIV. Effect of lycopene and zymosan on the complement level in rabbit serum. The values indicate the relative complement levels (initial values = 1) 5 days after the intravenous injection of 10 mg lycopene or 75 mg zymosan.

In another series of experiments the effect of lycopene and zymosan on the complement levels of rabbits were compared. Determinations were made both of the total complement and of its individual components. Only relative values were determined since only the possible fluctuations in the complement level from one time to another were of interest in the present connection. The complement levels were determined 5 days after the treatment

of the animals. Table XIV shows the results of a typical experiment. With both lycopene and zymosan, the complement levels revealed an increase, especially that of the C'1 component.

One of the most characteristic properties of substances inducing an increased unspecific resistance seems to be their ability to adsorb properdine *in vitro* when added to blood serum at + 17° C (Pillemer, Blum, Lepow, Ross, Todd and Wardlaw, 1954; Pillemer, Schoenberg, Blum and Wurz, 1955). This property was found not to be shared by lycopene. Whether this is due to basic differences between the mechanisms of action of lycopene and other resistance-enhancing substances, or simply to the technical

				Percent hemolysis of PNH erythrocytes
Before injection				40
1 hours after injection				60
2 " " "				63
3 " " "				67
4 " " "				72
72 " " "				81

Table XV. Influence of lycopene on the effect of rabbit serum to hemolyse PNH erythrocytes after intravenous injection of 10 mg lycopene into the rabbit.

difficulty in getting lycopene dispersed without using Tween (the latter would render it difficult to spin down the lycopene after the treatment of the serum) is not yet known.

On the basis of the observation of Hinz, Jordan and Pillemer (1956) that properdin is involved in the system which hemolyses erythrocytes from patients suffering of paroxysmal nocturnal hemoglobinuria (PNH), the effect of rabbit serum on such cells has been studied *in vitro* in connection with lycopene injection. It was found that the capacity of serum to hemolyse PNH-cells increases already after one hour following the injection of lycopene, and reaches maximum after four hours. This level is then maintained during further three days. Table XV shows data on this effect.

IX. Effect of lycopene on the level of a resistance-enhancing erythrocyte-stroma factor.

The previous findings that bacteria grown on a lycopene-containing substrate produce a resistance-enhancing factor with lipophilic properties, and that lycopene, when injected into animals, induces an increased unspecific resistance, suggested that there might occur a factor in blood, the level of which could be correlated with the nonspecific resistance of the animal. Since it could be anticipated that such a factor, like that previously found in *S. lactis*, might exhibit lipophilic properties, attempts were made to extract serum from rabbits before and after lycopene-treatment by means of various organic solvents, and to investigate the capacity of these extracts to enhance unspecific resistance in mice.

Ethylacetate proved to be a suitable solvent for this purpose. 15 ml rabbit serum was shaken with three 5-ml portions of ethylacetate. The extracts were pooled and the ethylacetate was evaporated in a desiccator. The residue was suspended in 6 ml physiological saline, and aliquots of the suspension were injected into mice. Tables XVI and XVII show two typical experiments. In Table XVII, the effect of the blood factor on the resistance of mice against infection with *Klebsiella p.*, AD strain, is shown; in the experiment in Table XVIII, the resistance against Ehrlich ascites tumors was tested. In both cases, comparison is also made between the resistance-enhancing potencies of the blood factors obtained before and 5 days after lycopene-treatment of the rabbit from which the blood was taken. As seen in the Tables, in both cases the lycopene treatment increased the potency of the blood factors in enhancing the resistance of the mice against the challenge.

Injected substance	Survivors after		
	24	30	48
	hours following infection		
None	5	0	0
Blood factor from rabbit before lycopene-treatment corresponding to			
0.00025 ml serum	5	4	2
0.0025 " "	4	3	2
0.025 " "	6	3	3
0.25 " "	4	1	1
Blood factor from rabbit after lycopene-treatment corresponding to			
0.00025 ml serum	6	5	5
0.0025 " "	5	4	4
0.025 " "	6	5	5
0.25 " "	4	4	4
Lycopene, 1 mg	6	6	6

Table XVI. Effect of blood factor extracted from rabbit sera before and 5 days after intravenous treatment of the rabbit with 10 mg lycopene on the resistance of mice against *Klebsiella p. AD*.

In the course of these experiments it was observed that the yields of active factor were greater when the blood samples were hemolytic. This directed the attention to the erythrocytes as the possible sites of the active factor. Erythrocytes were isolated by centrifuging defibrinated blood, washed twice with saline, and suspended in five volumes of water. The hemolysate was extracted with ethylacetate as described above. These extracts were found to give effects, similar to those previously observed with the serum extracts, but at a much higher potency. The yield could

Injected substance	Survivors after days following inoculation of 1000 cells											30
	10	12	14	16	18	20	22	24	26	28		
None	6	5	5	5	4	2	1	1	0	0	0	
Blood factor from rabbit before lycopene	5	5	5	5	4	4	4	3	3	1	1	
Blood factor from rabbit after lycopene	6	6	6	6	5	5	5	5	5	5	4	
days following inoculation of 100 cells												
None	5	5	5	5	5	5	3	2	2	1	1	
Blood factor from rabbit before lycopene	6	6	6	6	6	5	4	3	3	1	1	
Blood factor from rabbit after lycopene	6	6	6	6	6	6	6	6	6	6	6	

Table XVII. Effect of a blood factor from rabbit before and after lycopene treatment on the resistance of mice against Ehrlich ascites tumor. 6 female mice in each group. Dose of factor, corresponding 0.25 ml serum per mouse, injected intraperitoneally 24 hours before the inoculation of the tumor cells.

further be increased by spinning down the stroma and using this rather than the whole hemolysate as starting material for the extract to be injected. Further modifications of the procedure

	Survivors after						
	3	5	7	15	20	24	48
hours following infection							
Extract from erythrocyte stroma hemolysed at 37° C for 24 hours	6	6	6	6	5	2	2
Dose corresponding to 0.5 ml blood/mouse							
Extract from erythrocyte stroma hemolysed at ± 0° C for 24 hours	3	2	0	0	0	0	0
Dose corresponding to 0.5 ml blood/mouse							
Control	4	2	0	0	0	0	0

Table XVIII. Effect of extracts of rabbit erythrocyte stroma, hemolysed at + 37° C and ± 0° C for 24 hours, on the resistance of mice against *Klebsiella* p. AD. 6 female mice in each group. The substances were given intraperitoneally 24 hours before challenge.

consisted in using acetate buffer rather than ethylacetate as extracting agent, and in introducing an incubation at 37° C for 24 hours of the hemolysate before the separation of the stroma.

The detailed final procedure was as follows: Rabbit erythrocytes, washed twice with saline, were suspended in 5 volumes of water, and the hemolysate was incubated at 37° C for 24 hours.

The stroma was isolated by centrifugation at 25,000 *g* for 30 minutes, washed 3 times with isotonic saline, and extracted 3 times, for 10 minutes each time, with 0.05 M acetate buffer (pH 5.5) at 100° C. Aliquots of the extracts so obtained were

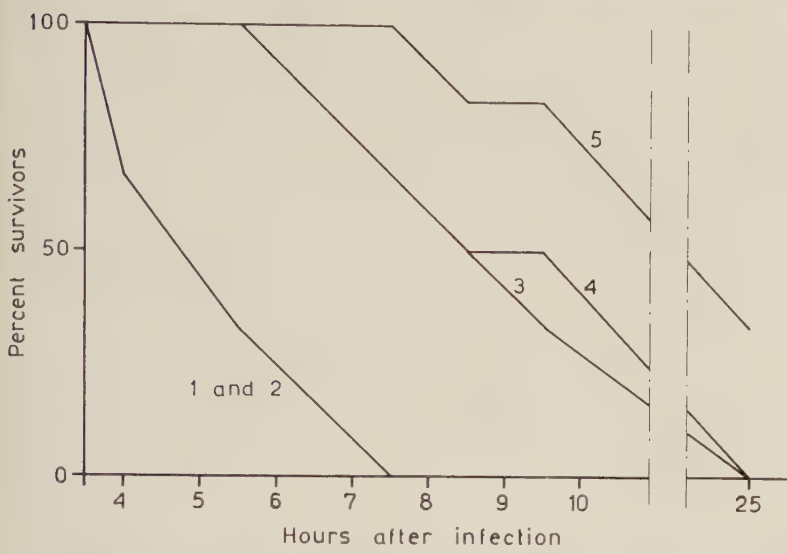


Fig. 9. Effect of extracts of rabbit erythrocyte stroma on resistance of mice against *Klebsiella p. AD*. 10 female mice in each group.

- 1: Control.
- 2: Blood from untreated rabbit. Dose of substance corresponding to 0.02 ml whole blood per mouse.
- 3: Blood from rabbit treated intravenously with 10 mg lycopene 5 days before taking the blood sample. Dose of substance corresponding to 0.02 ml whole blood per mouse.
- 4: Blood from untreated rabbit. Dose of substance corresponding to 0.4 ml whole blood per mouse.
- 5: Blood from rabbit treated as in 3. Dose 0.04 ml whole blood per mouse.

injected into mice. The importance of the incubation at 37° C is illustrated by the experiment shown in Table XVIII; when care was taken to keep the blood sample cold throughout the preparation no activity whatsoever could be obtained. In Fig. 9, a comparison is made between the potencies of stroma factor from rabbit before and 5 days after treatment of the rabbit with

10 mg lycopene. As can be deduced from the data in Fig. 9, the potency of the stroma factor is increased, roughly, by a factor of 10.

It is noteworthy that the procedure found to be optimal for the preparation of the stroma factor is closely similar to that described by Erslev A.J. (1957). for the preparation of the so-called erythropoietic factor. Whether the stroma extracts described herein contain lipopolysaccharides of the type recently described by Landy and Shear (1957) to occur in animal tissues is not yet established but appears probable.

X. Lycopene effect and the Schwartzmann phenomenon.

Landy and Shear (1957) have shown that bacterial lipopolysaccharides with a resistance-enhancing activity may be active also in evoking the so-called Schwartzmann phenomenon. This phenomenon consists in a local necrosis of the skin on an animal which has been treated intradermally with a certain substance,

Treated Jan. 24	Intradermal injection of evoker Jan. 28	Intravenous injection Jan. 29	Observed Jan. 30
—	0.1 ml	0.5 ml evoker	neg.
10 mg lycopene	0.1 ml	0.5 ml evoker	3 +
40 mg zymosan	0.1 ml	0.5 ml evoker	3 +
—	0.1 ml	10 mg lycopene	1 +
—	0.1 ml	100 mg zymosan	3 +

Table XIX. Effect of zymosan and lycopene on the Schwartzmann phenomenon. Two rabbits of 1.5 kg in each group.

e. g. a toxin, and received, 24 hours later, an intravenous injection of the same substance. In the present case, a broth-suspension of heat-killed *Klebsiella p.* (+70° C, 30 min.) was used for making the skin preparations. This proved to be a rather weak evoker of the Schwartzmann phenomenon. Thus, as shown in Table XIX, no necrosis occurred within 24 hours when only the

evoker was given intravenously. However, if the experiment was performed with rabbits which had received an intravenous injection of 10 mg lycopene 4 days before the skin preparation, a strong Schwartzmann phenomenon ensued upon the intravenous injection of the evoker. Moreover, a positive reaction could also be obtained if lycopene was given instead of the intravenous injection of the evoker. Zymosan (40 mg) had the same effect as lycopene.

XI. Some metabolic effects of injected lycopene.

In view of the shocklike effect which could be observed in rabbits given a high dose of lycopene, it seemed to be of interest to perform some simple laboratory tests on animals in connection with lycopene treatment.

Blood sugar determinations on rabbits revealed no deviations from the normal value, nor was lycopene treatment found to alter the glucose tolerance of rabbits. In mice, a dramatic decrease of the liver glycogen level could be recorded one hour after the intraperitoneal injection of lycopene. In one experiment, for example, where twelve mice were given lycopene and six served as controls, the control group had an average liver glycogen level of 6.5 percent, while the corresponding average value in the treated group was 1.7 percent one hour after the lycopene treatment.

In rabbits, a drastic rise in the number of granulocytes was observed, reaching a maximum 24 hours after the injection of lycopene; the total number of leucocytes showed no increase except in occasional instances. The sedimentation rate of erythrocytes was normal, as well as the prothrombin index. There was no change in the serum cholesterol level. The total serum protein, on the other hand, was often slightly elevated. However, paper electrophoresis revealed no clear-cut change in the relative concentration of the individual protein fractions.

XII. Discussion.

During the last decades a large number of substances have been described which, upon injection into animals, induce an increased nonspecific resistance against external challenge (Petersen, 1928; Perla and Marmorston, 1941; Kiser, Lindh and de Mello, 1956). Certain forms of colloidal sulfur and metals, zymosan, various bacterial and animal lipopolysaccharides, certain dextrans and levans, etc., and, as shown by the present work, lycopene, are examples of such substances.

In spite of a great chemical diversity, all these substances seem to exert their resistance-promoting activity according to a strikingly similar pattern. The most characteristic feature of this pattern is that the effect strictly depends on the time elapsed between the administration of the agent and the challenge, and that, depending on this time lapse, the obtained effect can be both beneficial and deleterious. It seems to be a rule, valid for all instances where this time-relationship has been studied, that the effect is optimal when the substance is given within the time range of 1–5 days before challenge, and that there is a critical period immediately after challenge where there is a risk for inducing, by giving some of these substances, a decrease rather than an increase in the animal's resistance.

Thus, in spite of the great chemical diversity among all these agents, it is difficult to escape the conclusion that their actions must ultimately rely upon a common principle or mechanism. Since among all these substances the recently described bacterial lipopolysaccharides (Landy, 1956; Landy and Pillemer, 1956 b) have the highest potency hitherto observed it appears logical to assure that these substances are closely related, possibly chemical analogs, to the principle which is responsible for the nonspecific resistance of the animal organism. That so may be the case is

also indicated by recent reports by Landy and Shear (1957) on the occurrence of resistance-enhancing lipopolysaccharides in animal tissues.

The role of lycopene in this connection is not yet fully understood. That its action may consist in a stimulation of the formation or release of lipopolysaccharides is indicated both by the early finding that the presence of tomato juice in the culture medium of streptococci increases the yield of resistance enhancing activity in the bacterial extracts, and by the more recent observation that extracts of rabbit erythrocyte stroma reveal an increased potency in enhancing resistance in mice if the rabbits have been treated with lycopene.

Beyond these indications, however, there are no clear terms as yet available to explain in which way lycopene may exert such an action. One conceivable explanation might be that lycopene in some way is metabolized in the animal organism, and that some of its metabolic products enter as parts in the lipopolysaccharide complexes responsible for the resistance of the organism. There are no known ways, however, in which lycopene could be thought to undergo metabolic reactions in animals. Preliminary experiments indicate, in fact, that the injected lycopene can be almost quantitatively recovered from an animal several days after injection. Moreover, the fact that squalene, which is chemically closely related to carotinoids, and is an active metabolite in animal tissues, participating in the synthesis of cholesterol (Friedman, Byers and St. George, 1956), is completely inactive in promoting resistance, seems to further rule out the possibility that lycopene exerts its resistance-enhancing action by being directly metabolized.

A striking feature of the lycopene effect is its high degree of specificity. Not only close relatives of lycopene, such as e. g. β -carotene, were found to be much less active than lycopene itself, but the same was also valid when the *all-trans*-form of lycopene was converted into the *cis*-isomer forms. In general, it would appear that, in spite of the great chemical diversity among the individual substances which are known to promote unspecific resistance, there is a remarkable requirement of steric specificity within each group of such substances. Thus, the concept

of steric specificity has repeatedly been emphasized in the case of polysaccharides, where the degree of branching seems to be of outstanding significance for the biological activity (Pillemer, Blum, Lepow, Wurz and Todd, 1956), and even in the case of such a simple substance as colloidal sulfur there is reason to believe that some sort of a steric configuration may play a certain role. This is indicated by the data of Konowalchuk, Hinton and Reed (1954) and of Kiser, Lindh and de Mello (1956) that only sulfur prepared in a certain manner gives satisfactory biological activity.

These facts point to the possibility that we here have to deal with a reaction mechanism that requires that the injected substance, in order to be active, fit into a given pattern, possibly a specific cellular structure, which, when thus activated, enables the cell to an enhanced production or release of lipopolysaccharides. It may be hoped that by having obtained in lycopene a chemically well-defined and relatively easily available agent with a high promoting effect on nonspecific resistance against a broad spectrum of external challenges, the possibilities have been widened for a future study of this mechanism.

SUMMARY

The effect of lycopene in enhancing nonspecific resistance in animals has been studied. The studies were based on the observation that the addition of tomato juice to the culture medium of *Streptococcus lactis* stimulates the production of resistance-enhancing principles in these bacteria, and that this effect of tomato juice is due to lycopene.

Lycopene, natural or synthetic, was suspended in Tween-80, the suspension diluted with physiological saline, and injected into mice or rabbits. The following effects of injected lycopene were studied:

1) 1 mg *all-trans* lycopene, injected intraperitoneally into a mouse 24 hours before infection with a massive dose of *Klebsiella pneumoniae* induces a high degree of resistance against the infection. *Cis*-isomers of lycopene, carotene, crocetin, or bixin are much less efficient in inducing this effect, while phytoene, squalene and vitamin A are completely inactive (Chapter III).

2) The resistance-enhancing effect of *all-trans*-lycopene is dependent on the dose of lycopene and/or bacteria, and on the time elapsed between the injection of lycopene and the infection. High doses of lycopene are toxic, rabbits being more susceptible than mice. The lycopene effect is typically preventive, optimal effects being obtained when lycopene is given 24 hours before infection. The effect lasts for about 72 hours but can be extended by exposing the animal to challenge. The intravenous and intramuscular routes of injection are likewise effective, whereas lycopene given *per os* is without effect (Chapters IV—V).

3) Irradiation of mice with an X-ray dose of 100—1000 r, given 24 hours after the injection of lycopene, does not eliminate the resistance-enhancing effect of lycopene; at a dose of 3000 r, the lycopene effect is completely abolished. Also low doses of X-ray can eliminate the effects of lycopene in enhancing antibacterial

resistance, provided that the lycopene is given at least 48 hours after irradiation (Chapter VI).

4) Lycopene can protect mice against the development of Ehrlich ascites tumors, provided that the tumor has not exceeded a size of 10,000 cells at the time of the lycopene injection (Chapter VII).

5) Injected lycopene causes an elevation of the serum properdin level, as well as of the levels of all four complements. Yet, there is no clear correlation between these effects of lycopene and those on the nonspecific antibacterial resistance of the animal. Lycopene exhibits no antibacterial activity *in vitro*, nor does it adsorb properdin when incubated with serum at $+17^{\circ}\text{C}$ (Chapter VIII).

6) Extracts of rabbit erythrocyte stroma, when injected into mice, enhance the nonspecific resistance of the animals against bacterial infections and Ehrlich ascites tumor. The potency of the stroma extract in exhibiting this effect increases upon treatment of the rabbit, from which the extract is prepared, with lycopene five days prior to taking the blood sample (Chapter IX).

7) Lycopene promotes the manifestation of the Schwartzmann phenomenon as evoked by a broth suspension of heat-killed *Klebsiella pneumoniae*. (Chapter X).

The findings are discussed in terms of the hypothesis that lycopene, by a physico-chemical action on certain cellular structures, enhances the production and or release of tissue lipopolysaccharides apted for the defence of the animal organism against various forms of external challenge.

ACKNOWLEDGEMENT

I am greatly indebted to Professor Olov Lindberg and Dr. Lars Ernster of the Wenner-Gren Institute, University of Stockholm, for continuous support and encouragement throughout these studies. My thanks are also due to Dr. Arne Forssberg, Institute for Radiophysics, Professor Georg Klein, Dept. of Tumor Biology, and Professor Berndt Malmgren, Institute of Bacteriology, Karolinska Institutet, Stockholm, for valuable help in connection with certain parts of this investigation.

The initial part of these studies has been conducted at Kronprinsessan Lovisas Barnsjukhus: to the Director of this Hospital, Professor Curt Gyllenswärd, I will express my deep gratitude. Greatful acknowledgement is also due to Mrs. Kajsa Lennfalk, Mrs. Birgitta Grepe and Mrs. Ingalill Lindgren for valuable technical assistance.

The work has been aided by grants from "Therese och Johan Anderssons Minne", "Nationalföreningen mot Tuberkulos", "Byggmästare C. O. Lundbergs Minnesfond", "Atomkommittén" and "Naturvetenskapliga forskningsrådet".

References

Birch-Hirschfeld, L., 1939: Proteolytische Fermentsekretion pyogener Staphylococcen und ihre pathogenische bedeutung. *Klin. Wschr.* 18, 831.

Boyd, W. C., 1956: Fundamentals of immunology. Interscience publishers, Inc., New York.

Dixon, F. J., Talmage, D. W. and Maurer, P. H., 1952: Radiosensitive and radioresistent phases in the antibody response. *J. immunol.*, 68, 639.

Erslev, A. J., 1957: L. Observation on the nature of the erythropoietic serum factor. *Journ. of Lab. and Clin. Med.* 50, 543.

Francis, T. Jr, 1948: Response of the host to the parasite, in Dubos, R. J., *Bacterial and mycotic infections of man* (J. B. Lippincott Company, Philadelphia) p. 90.

Friedmann, M., Byers, S. O., and St. George, S., 1956: Cholesterol metabolism. *Ann. Rev. Biochem.*, 25, 613.

Gillam, A. E. and Kon, S. K., 1940: The passage of carotenoids from food to milk in the cow. The fate of lycopene. *J. of Dairy Research* 11, 266.

Hinz, C. F., Jordan, W. S., and Pillemer, L., 1956: The properdin system and immunity. The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. *J. Clin. Invest.* 35, 453.

von Isler, O., Gutmann, H., Lindlar, H., Montavon, M., Rüegg, R., Ryser, G., and Zeller, P., 1956: Synthesen in der Carotinoid-Reihe. *Helv. Chim. Acta* 54, 463.

Karrer, P. and Jucker, E., 1956: Carotinoide. Verlag Birkhäuser Basel.

Kiser, J. S., Lindh, H., and de Mello, G. C., 1956: The effect of various substances on resistance to experimental infections. *Ann. N. Y. Acad. Sci.*, 66, 312.

Konowalchuk, J., Hinton, N. A., and Reed, G. B., 1954: Antibacterial action of reaction product of cysteine and iron. *Can. J. Microbiol.* 1, 175.

Landy, M., 1956: Increase in resistance following administration of bacterial lipopolysaccharides. *Ann. N. Y. Acad. Sci.*, 66, 292.

Landy, M., and Pillemer, L., 1956 a: Elevation of properdin levels in mice following administration of bacterial lipopolysaccharides. *J. Exptl. Med.*, 103, 823.

Landy, M., and Pillemer, L., 1956 b: Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides. *J. Exptl. Med.*, 104, 383.

Landy, M., and Shear, M. J., 1957: Similarity of host responses elicited by polysaccharides of animal and plant origin and by bacterial endotoxins. *J. Exptl. Med.*, 106, 77.

Lingen, C., 1952: Experimentella studier över antibiotica och tuberkulos. Serologiska studier, *Nordisk Medicin* 48, 1625.

Lingen C., Lindberg, A., and Ernster, L., 1955: Studies on a factor promoting biological resistance. *Exptl. Cell Research Suppl.* 3, 244.

Lingen, C., Melin, K.-A., and Enell, H., 1950: Diplomycinbehandling vid tuberkulös meningo-encephalit. *Ugeskrift for Læger*, 112/41, 1416.

Marcus, S., Donaldson, D. M., and Esplin, D. W., 1955: Role of host defenses in systemic *Klebsiella Pneumoniae* infection: Effect of antibiotics in normal and X-irradiated mice. *J. Immunol.* 74, 494.

Nungester, W. J., 1954: Nonspecific factors in immunity. *Ann. Rev. Microbiol.*, 8, 363.

Perla, D., and Marmorstone, J., 1941: Natural resistance and clinical medicine. Little, Brown and Co., Boston, Mass. p. 1227.

Petersen, W. F., 1928: Non-specific protein therapy. *In* newer knowledge of bacteriology and immunity. Univ. Chicago Press. Chicago, Ill. p. 1086.

Pillemer, L., Blum, L., Lepow, I. H., Wurcz, L., and Todd, E. W., 1956: The properdin system and immunity. *J. Exptl. Med.* 103,1.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., 1954: The properdin system and immunity: 1. Demonstration and isolation of a new serum protein, properdin and its role in immune phenomena. *Science*. 120, 279.

Pillemer, L., Schoenberg, M. D., Blum, L., and Wurcz, L., 1955: Properdin system and immunity: 2. Interaction of the properdin system with polysaccharides. *Science*. 122, 545.

Sandeval, A., and Zechmeister, L., 1949: Biochemical preparations. J. Wiley and Sone, Inc. New York. Vol. 1. p. 57.

Schrek, R., 1936: A method for counting the viable cells in normal and in malignant cells suspensions. Am. J. Cancer 28, 389.

Trombly, H.H., and Porter, J.W., 1953: Arch. Biochem. and Biophysics 43, 443.

MASTERPRINT
FRIDMANS BOKTRYCKERI AB
STOCKHOLM 1958